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## Comparison of reversed-phase and ion-pair chromatography for the determination of strychnine in animal tissues

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## Comparison of reversed-phase and ion-pair chromatography for the determination of strychnine in animal tissues

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### Abstract

Ion-pair and reversed-phase high-performance liquid chromatography (HPLC) were evaluated for quantification of strychnine in mountain beaver tissues. Retention time shifts hindered strychnine quantification with both HPLC systems. Co-extracted free fatty acids released during storage formed ion-pairs with strychnine, resulting in increased retention by reversed-phase HPLC. Competition with co-extracted basic compounds is likely responsible for the decreased retention of strychnine by ion-pair HPLC. Following an acid-base clean-up, optimal results were obtained with reversed-phase HPLC. Ion-pair chromatography was then used for qualitative confirmation of strychnine residues.

### 1. Introduction

Strychnine has been used as a toxicant for the management and control of vertebrate pests such as rats and other rodents. It is usually administered through the use of commercial grain bait carriers. Our laboratory has been conducting studies to determine the efficacy of strychnine for the control of mountain beavers (*Aplodontia rufa*). For wildlife pest control applications, administration of strychnine by carriers is currently restricted to below-ground applications to minimize exposure to non-target species. However, strychnine residues in target animals could result in secondary poisoning of non-target species. To evaluate potential secondary

hazards, a reliable analytical method was needed to determine low levels of strychnine in various animal tissues. In order to provide analytical chemistry support for Environmental Protection Agency efficacy and secondary hazard Federal Insecticide, Fungicide and Rodenticide Act registration studies, our laboratory required a sensitive method to quantify strychnine residues in combined muscle–liver–kidney (MLK) tissues and gastrointestinal tracts (GIT) of mountain beavers at levels less than 1 µg/g.

Gas chromatography (GC) with flame ionization detection [1,2] and thermionic nitrogen–phosphorous detection [3–5] has been used for the detection of strychnine in relatively clean matrices such as grain baits. As normal- [6,7] and reversed-phase [8,9] HPLC methods have been used for the determination of strychnine in

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a variety of mammalian tissues, we concentrated on the development of an HPLC method to fulfil our analytical needs. As we expected strychnine to be more polar than most of the potentially co-extracted chromatographic interferences from the biological sample matrices, we assumed a reversed-phase HPLC system would be better than normal-phase HPLC to quantify strychnine in mountain beaver tissue. We attempted to develop a reversed-phase HPLC system in which strychnine would elute before the co-extracted interfering compounds. Ion-pair HPLC was suitable for strychnine quantification in MLK samples but was unacceptable for GIT samples due to retention time irregularities. Reversed-phase HPLC with a phosphate-buffered mobile phase was suitable for quantification of strychnine in GIT extracts but gave unacceptable variations for the retention time of strychnine in MLK extracts. The purpose of this study was to determine the cause of the retention shifts associated with both HPLC systems and to develop a single HPLC method for the quantification of strychnine in both mountain beaver MLK and GIT tissue.

## 2. Experimental

### 2.1. Apparatus

For chromatography we used a Hewlett-Packard Model 1090M liquid chromatograph (Hewlett-Packard, Avondale, PA, USA) equipped with a temperature-controlled column oven, an ultraviolet diode array detector (DAD) and a 25 cm  $\times$  4.6 mm I.D., 5- $\mu$ m Octyl-H (Keystone Scientific, Bellafonte, PA, USA) HPLC column. A horizontal mechanical shaker with a stroke of 2 3/8 inch (1 inch = 2.54 cm) (Eberbach, Ann Arbor, MI, USA) was used.

### 2.2. Reagents

All solvents were HPLC grade. The strychnine alkaloid standard was 98% (Aldrich, Milwaukee, WI, USA). Stock standard was 1 mg/ml in methanol; calibration standards, 0.2–50  $\mu$ g/ml

in methanol. The endogenous constituents: bile salts, bilirubin, oleic acid, linoleic acid, tri-linolein, phosphatidyl choline, phosphatidyl inositol, myoglobin, lactic acid, leucine, tyrosine, glutamic acid and lysine were obtained from Sigma (St. Louis, MO, USA)

The heptanesulfonic acid ion-pair mobile-phase solution (2.4 mM) was prepared by adding 12.5 ml of ion-pair reagent IPC B7 (Alltech, Deerfield, IL, USA) to 1 l of water, followed by the addition of 60 ml of methanol. The pH of the resulting aqueous solution was about 3.2. The phosphate-buffered mobile-phase solution (25 mM) was prepared by adding 3.4 g of monobasic potassium phosphate (Mallinckrodt, Paris, KY, USA) to 1 l of water. The pH of the resulting aqueous solution was about 4.8.

### 2.3. Sample preparation

Muscle–liver–kidney and homogenized gastrointestinal tract mountain beaver tissues were obtained from Denver Wildlife Research Center's Olympia field station (Olympia, WA, USA). Muscle, liver and kidney (30:58:12) were combined and blended till homogeneous. Accurately weighed tissue aliquots of 3.0 g were transferred to 150  $\times$  25 mm 50-ml screw-cap (polyethylene-lined) borosilicate glass tubes and spiked with aliquots of the stock strychnine standard to give a final concentration of 0.25, 0.5, 5 or 50  $\mu$ g strychnine/g tissue. Controls were not spiked with strychnine.

### Sample extraction

Methanol (1 ml) was added to each sample. The samples were vortexed for 30 s, followed by the addition of 1 ml 5 M NaOH. The samples were mixed with a vortex mixer for another 30 s and allowed to stand for 5 min. *n*-Butyl chloride (15 ml) was added followed by 30 s of vortex-mixing. Samples were shaken on the mechanical horizontal shaker for 10 min, vortex-mixed for 30 s, and centrifuged for 5 min at 1000 *g*. The top *n*-butyl chloride layer was transferred by decanting for the MLK samples and aspirating for the GIT samples to a clean 50 ml glass screw-cap tube. The extraction was repeated two

more times with 10 ml each *n*-butyl chloride. Appropriate *n*-butyl chloride extracts were combined.

Tissues were spiked and extracted in lots of seven replicates. Two lots of the combined *n*-butyl extracts of tissue samples were placed in a hot water bath at approximately 75°C and evaporated to dryness under a gentle stream of nitrogen. One of these lots was reconstituted in mobile phase and analyzed by ion-pair HPLC and the other reconstituted in methanol and analyzed by reversed-phase HPLC as specified under HPLC analysis.

#### *Acid-base clean-up*

The other two lots of combined *n*-butyl chloride extracts were taken through an acid-base clean-up procedure. For this clean-up, a quaternary ammonium form of strychnine was favored by the addition of 10 ml of 0.5 *M* HCl to the *n*-butyl chloride extract. The mixture was shaken on a horizontal mechanical shaker for 10 min, centrifuged 3 min, and the top *n*-butyl chloride layer discarded. Any emulsion was left with the aqueous layer. This *n*-butyl chloride extraction of non-ionic (acidic) compounds was repeated twice. The neutral form of strychnine was favored by the addition of 2 ml of 5 *M* NaOH and vortex-mixing. *n*-Butyl chloride (20 ml) was added, the sample vortex-mixed, shaken on the horizontal mechanical shaker for 10 min, centrifuged for 3 min and the strychnine containing *n*-butyl chloride transferred with a pipet to a clean 50-ml screw-cap tube. The extraction was completed two more times with 10 ml each of *n*-butyl chloride, combining the *n*-butyl chloride from each extraction. The combined *n*-butyl chloride extract was placed in a hot water bath (75°C) and evaporated to dryness with a gentle stream of nitrogen. One of these lots was reconstituted in mobile phase and analyzed by ion-pair HPLC and the other reconstituted in methanol and analyzed by reversed-phase HPLC as specified under HPLC analysis.

#### *Endogenous co-extractants*

To evaluate the effects of potentially co-extracted various endogenous constituents on the

chromatography of strychnine, 250 mg of each endogenous constituent was combined with 15 µg strychnine in 300 µl methanol and 1 ml water. A 1 ml aliquot of 5 *M* NaOH was added to this mixture which was processed identically to the strychnine-spiked tissue samples. Aliquots of each mixture were analyzed by reversed-phase and ion-pair HPLC both with and without the acid-base clean-up procedure.

#### *2.4. HPLC analyses*

The dried tissue extracts were reconstituted in either 3.0 ml 70% 2.4 mM heptanesulfonic acid–30% acetonitrile or methanol for analysis by ion-pair or phosphate-buffered HPLC, respectively. Reconstitution was aided by placing the solution in an ultrasonic water bath for 15 min. The solutions were then vortexed and filtered through a disposable 0.45 µm Teflon syringe filter and into an LC vial for analysis.

#### *HPLC conditions*

For both HPLC conditions a Hewlett-Packard 1090M liquid chromatograph and a 25 cm × 4.6 mm I.D., 5 µm Octyl-H HPLC column was used at a temperature of 40°C with a mobile-phase flow-rate of 1 ml/min, a detector wavelength of 254 nm and an injection volume of 25 µl.

The mobile-phase parameters used for the ion-pair reversed-phase and phosphate-buffered reversed-phase systems are summarized in Table 1. An isocratic mobile phase was used for elution of strychnine. Following elution of strychnine, a high percentage organic modifier mobile phase was used as a column wash to remove appreciable amounts of late eluting non-polar material.

### **3. Results and discussion**

Ion-pair reversed-phase HPLC was found to be well suited for strychnine quantification in the MLK samples. Even though no buffer was used with the ion-pairing reagent, the pH 3 mobile phase was sufficiently acidic to ensure that strychnine would be predominantly ionized and capable of forming an ion-pair with hepta-

Table 1  
LC mobile phase parameters

LC parameters	Ion pair (MLK)	Phosphate buffer (GIT)
Mobile-phase composition	70% A (2.4 mM heptanesulfonic acid, pH 3.2) 30% B (acetonitrile)	70% A (25 mM monobasic potassium phosphate, pH 4.8) 20% B (acetonitrile) 10% C (methanol)
Run time	15 min	10 min
Column wash	5-min gradient to 15:85 (A–B), hold 5 min	5-min gradient to 5:75:10 (A–B–C), hold 10 min
Column equilibration	5-min gradient to 75:25 (A–B), hold 5 min	5-min gradient to 70:20:10 (A–B–C), hold 10 min

nesulfonate. An excellent linear correlation was obtained between detector response and strychnine concentration for calibration standards ranging from 0.2  $\mu\text{g/ml}$  to 50  $\mu\text{g/ml}$  ( $r^2 = 0.9999$ ). Table 2 shows the recoveries of strychnine from tissues fortified at four different levels in replicates of seven. These data show that *n*-butyl chloride served very well as an extractant for strychnine from tissue as indicated by lack of emulsions during extraction and recoveries of 94% with an average relative standard deviation (R.S.D.) of 4%. Furthermore, these recoveries as well as the excellent recoveries obtained during method development, both with and without the acid-base clean-up, indicate that strychnine did not form any quaternary artifacts with *n*-butyl chloride which may have negatively affected analyte recovery. The

acid-base clean-up had no effect on the chromatography of strychnine in the MLK samples when analyzed by the ion-pair reversed-phase HPLC system.

Ion-pair HPLC was found to be unsuitable for GIT samples even when samples were taken through the acid-base clean-up procedure. When fortified GIT sample extracts were analyzed by ion-pair LC, the retention time of strychnine appeared to be approximately 1.5 min before the retention time observed for the external standard. Addition of strychnine directly to an extract resulted in enhancement of the early peak. The UV spectrum of the early peak matched that of strychnine, verifying the shift in retention time in the presence of sample matrix. Because the samples were taken through an acid-base clean-up step which removes acids, the matrix interfer-

Table 2  
Recovery of strychnine from fortified mountain beaver tissue

Fortification level ( $\mu\text{g/g}$ )	Mean recovery ( $n = 7$ ) (%)					
	Ion-pair MLK		Reversed-phase MLK		Reversed-phase GIT	
	Mean	R.S.D.	Mean	R.S.D.	Mean	R.S.D.
0.25	95	7	73	2	82	11
0.50	89	4	77	1	80	9
5.00	97	4	86	1	96	0.9
50.0	94	2	90	6	99	0.6
Average	94	4	82	3	89	5

ants were presumably basic compounds. Large amounts of basic compounds in this dirty sample matrix could compete with strychnine for the ion-pairing agent and promote greater partitioning of strychnine in the mobile phase than on the stationary phase, resulting in earlier elution of strychnine. Despite varying the pH and organic–aqueous composition of the mobile phase, identical retention times for strychnine in samples and in the external standards could not be obtained. Quantification of strychnine in the GIT extracts by ion-pair reversed-phase HPLC was not possible.

In an effort to obtain satisfactory chromatography of strychnine in the cleaned-up GIT sample extracts, a mobile phase buffered with monobasic potassium phosphate was evaluated. Under these reversed-phase HPLC conditions, there was no competition between strychnine and co-extracted basic constituents for acidic ion-pairing reagents. With this phosphate-buffered system, the retention time shifts that occurred with the ion-pair system were not observed. For this phosphate-buffered reversed-phase HPLC system, linear regression analysis of calibration data for detector response versus strychnine concentrations of standards ranging from 0.2  $\mu\text{g/ml}$  to 50  $\mu\text{g/ml}$  showed a correlation coefficient of 0.9999. A slope of 1.010 and a correlation coefficient of 0.9999 were obtained for linear regression analysis of log response versus log concentration data. Recoveries of strychnine from cleaned-up GIT control samples fortified at four different levels in replicates of seven averaged 89% with an average R.S.D. of 5% (Table 2).

As it would be preferable to have one HPLC system for analysis of both types of samples, the MLK extracts were analyzed with the phosphate-buffered HPLC system. The retention time of strychnine in the MLK extract analyzed without the acid-base clean-up was approximately 0.5 min later than that of a strychnine standard. When the MLK extracts were subjected to the acid-base clean-up, the retention times for strychnine in the tissue extracts and of the external standard were identical. As the clean-up removed acids, the increased retention time in

the non-cleaned-up samples was probably due to ion pairing of endogenous organic acids in the sample matrix with strychnine. This strychnine–organic acid complex would be less polar than strychnine alone and retained to a greater extent on a reversed-phase HPLC system. Quantification of strychnine residues in both GIT and MLK samples was possible with the acid-base clean-up followed by phosphate-buffered reversed-phase HPLC. Recoveries of strychnine from the cleaned-up MLK control samples fortified at four different levels in replicates of seven averaged 82% with an average R.S.D. of 3% (Table 2). Fig. 1 shows reversed-phase HPLC chromatograms of a 0.20  $\mu\text{g/ml}$  strychnine standard, and control and 0.25  $\mu\text{g/g}$  strychnine fortified cleaned-up GIT and MLK tissue extracts. The lack of extraneous analyte-related peaks in these chromatograms as well as the fact that strychnine recoveries with the acid-base clean-up during method development approached 100%, suggest that strychnine did not ion-pair with  $\text{Cl}^-$  in the acidified aqueous phase. If strychnine had formed an ion-pair with  $\text{Cl}^-$  during this procedure, then strychnine would have partitioned into and been discarded with the organic phase.

None of the endogenous compounds affected the ion-pair HPLC retention time of strychnine. The carboxylic acid moiety of linoleic and oleic acids, the most common free fatty acids in meat ( $\text{pK}_a > 5$ ) and phosphoric acid moiety of the abundant phospholipid phosphatidyl choline and phosphatidyl inositol ( $\text{pK}_a$  1–2) are less acidic than the heptane sulfonic acid ( $\text{pK}_a < 0.5$ ) ion-pairing reagent present in the mobile phase. This suggests that the heptane sulfonic acid out-competed any of the other endogenous compounds for strychnine's nitrogen ion-pairing site and no retention time shift was noted.

Phosphatidyl inositol, oleic acid and linolenic acid were the only endogenous constituents which affected the reversed-phase retention time of strychnine. Both free fatty acids increased the reversed-phase retention time of strychnine by approximately 10%, indicating the formation of a fatty acid–strychnine complex which was less polar than strychnine. Following the clean-up,

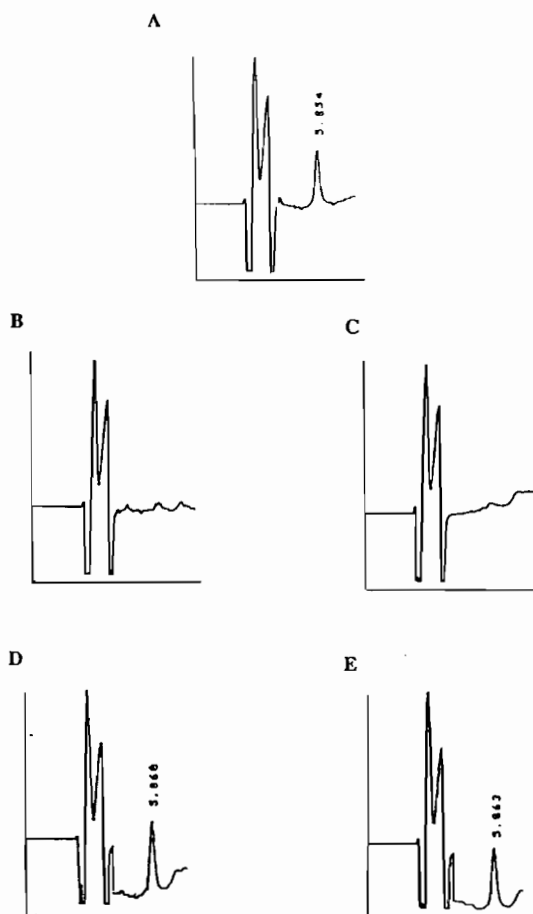


Fig. 1. Reversed-phase HPLC with ultraviolet detection [detector response vs retention time (min)] of 0.20 µg/g strychnine standard (A), control gastrointestinal tract (B) and muscle–liver–kidney (C), and spiked 0.25 µg strychnine/g gastrointestinal tract (D) and muscle–liver–kidney (E). Both the y- and x-axis scales are identical for all chromatograms.

the strychnine in this sample had the same retention time as the strychnine standard. The acid-base clean-up procedure was sufficiently acidic to disrupt the fatty acid–strychnine complex and permit fatty acid partitioning into *n*-butyl chloride. In this acidic environment, strychnine was protonated to form a quaternary amine and partitioned into the aqueous phase. Phosphatidyl inositol, which contains an ionizable phosphate moiety ( $pK_a$  1–2) also formed an ion pair with strychnine which similarly increased its reversed-phase retention time. However, this

retention time shift was still evident after the acid-base clean-up, indicating that the clean-up was not sufficiently acidic to dissociate the phosphatidyl inositol–strychnine complex.

These data indicate that free fatty acids are probably the endogenous constituents which are responsible for the strychnine reversed-phase retention time shift noted in the samples which had not been cleaned up via the acid-base clean-up. This phenomenon has not been observed in fresh tissues, but becomes evident after several months of frozen storage. Similarly, ion pairing of strychnine with endogenous acids has been attributed to peak splitting in the ion-pair HPLC analysis of strychnine residues in extracts of spoiled tissues [10]. Initially, most of the lipid material in animal tissue consists of triglycerides. However, during frozen storage, lipases release free fatty acids from triglycerides [11]. As the free fatty acid content of meat increases during storage, more free fatty acids are available to ion-pair with strychnine, resulting in altered chromatographic behavior. However, we have demonstrated that an acid-base clean-up can be employed to dissociate the free fatty acid–strychnine ion pair, remove the fatty acids, and restore the normal strychnine chromatographic behavior.

The acid-base clean-up of MLK samples was not necessary with the ion-paired HPLC system because the concentration of acidic ion-pairing reagent out competed the endogenous acids for the basic sites on strychnine. Thus, under these ion-pairing conditions, the presence of the co-extracted endogenous acids had no effect on the retention of strychnine. However, co-extraction of presumably basic constituents prohibited the quantification of strychnine in extracts of fortified GIT tissue.

The R.S.D. for recoveries of strychnine from fortified samples was generally larger at lower concentrations than at higher concentrations. The larger R.S.D. at lower concentrations was attributable to finite experimental error and variable background contributions from the sample matrix. A small background response was observed near the retention time of strychnine in some of the control samples. The background



response, which would interfere proportionately more at lower concentrations, was generally less than 10% of the response of the lowest fortified concentration level.

Method limits of detection (MLOD) were determined from the average strychnine response obtained for samples fortified at the lowest concentration, and the average background response obtained at the retention time of strychnine for control samples. The MLOD, based on the strychnine concentration equivalent to three times the average background response for the reversed-phase phosphate-buffered HPLC conditions were 0.08  $\mu\text{g/g}$  for the MLK samples and 0.1  $\mu\text{g/g}$  for GIT samples.

The results of these studies show that reversed-phase HPLC can be used with an acid-base clean-up procedure to determine the presence of low levels of strychnine in animal tissues. By the addition of an external standard to strychnine-containing samples, the ion-pair system can then be used for the qualitative confirmation of these strychnine residues. This clean-up and HPLC procedure should also be applicable to the analyses of a wide range of basic drugs (i.e. alkaloids, barbiturates) and pesticides (i.e. paraquat, diquat) in stored tissues as is typical in many forensic, food science, agricultural and medical studies.

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